# Induced Pluripotent Stem Cell-based *in vitro* Modeling of the Osteogenesis and Chondrogenesis of Juvenile Osteochondritis Dissecans

A Thesis
Presented to
The Academic Faculty

By

Catriana C. Nations

In Partial Fulfillment
of the Requirements for the Degree
Bachelor of Science in Biomedical Engineering in the
College of Engineering

Georgia Institute of Technology
May 2019

Induced Pluripotent Stem Cell-based *in vitro* Modeling of the Osteogenesis and Chondrogenesis of Juvenile Osteochondritis Dissecans

Approved by:

Dr. Robert Guldberg, Advisor
Knight Campus for Accelerating Scientific Impact
University of Oregon

Dr. Andrés García
George W. Woodruff School of Mechanical Engineering
Georgia Institute of Technology

Date Approved: April 17, 2019

# ACKNOWLEDGEMENTS

I wish to thank Dr. Robert E. Guldberg and Giuliana E. Salazar-Noratto for this opportunity and their mentorship throughout this project. I would also like to thank the Undergraduate Research Opportunities Program for providing part of the funding for part of this project through the Presidential Undergraduate Research Award.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| JOCD | Juvenile Osteochondritis Dissecans |
| OCD | Osteochondritis Dissecans |
| iPSC | induced pluripotent stem cells |
| iMSC | induced mesenchymal stem cells |
| IHC | Immunohistochemistry |
| PCR | Polymerase chain reaction |
| FACS | Fluorescence-activated cell sorting |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| GAG | Glycosaminoglycan |
| ECM | Extracellular Matrix |
| Col1 | Collagen Type 1 |
| Col2 | Collagen Type 2 |

# ABSTRACT

The application of pluripotent stem-cell based *in vitro* models has become increasingly popular in medical research, especially for situations in which animal modeling is not sufficient to accurately describe the condition or for diseases where there is little research demonstrating the relationships between disease phenotype, pathological cellular mechanisms, and gene expression. Such is the case with Juvenile Osteochondritis Dissecans (JOCD), a degenerative bone disease that predominately affects the knee joints of children and progresses to early onset osteoarthritis. Previous research has involved the use of animal models or diseases similar to JOCD, but there has been little to no focus on the cellular mechanisms of this condition. Therefore, this study aimed to elucidate the cellular pathophysiology of JOCD as well as provide a test bed for future therapeutic interventions. We hypothesized that our iPSC *in vitro* models of JOCD would show protein dysfunction and accumulation in the rough endoplasmic reticulum as a hallmark of the disease, as previously shown in familial and equine OCD.

# CHAPTER 1

# INTRODUCTION

Juvenile Osteochondritis Dissecans (JOCD) is a disease involving the fragmentation of subchondral bone with no evidence of acute trauma, and it occurs primarily in the knee joint. Affecting mainly children and young adults, JOCD is becoming increasingly common and progresses into early onset osteoarthritis. As with many musculoskeletal diseases, the diagnosis of JOCD is highly dependent on imaging to identify lesions in the subchondral bone, and it is very difficult to ascertain whether a lesion is due to the disease or another cause. Once diagnosed, the general consensus of treatment options is based on either skeletal maturity or lesion stability, and little success has been reported (Edmonds & Polousky, 2013). The pathogenesis of the disease family, Osteochondritis Dissecans (OCD), is relatively well understood, but only relative to the tissue as a whole because there is little mention of cellular models in literature (Bruns et al., 2017).

Although very few models of JOCD exist, Familial Osteochondritis Dissecans (FOCD) and OCD have been modeled *in vitro* with a focus on cellular pathology, demonstrating that a model of JOCD could have potential to characterize the disease. In 2008, Garvican et al. found a significant difference between OCD and normal chondrocytes in matrix protein expression, suggesting that future models of diseases in the OCD may find similar results. This led to Xu et al. creating a cellular model of the chondrogenesis of the disease in 2016, and they were able to characterize a major aspect of the disease from their results. They found a similar correlation between FOCD and the gene expression of matrix proteins. However, both models neglected to incorporate osteocytes into their models, which are important to accurately model the knee joint, especially for adolescent joints that are still developing. Because the lesions of JOCD start in the

subchondral bone, there may be an underlying pathogenesis in the endo-ossification of the tissue that contributes to its fragmentation, and the exact pathogenesis of JOCD cannot be completely assumed from the behavior of other diseases, leaving this area open for investigation.

Induced pluripotent stem cells (iPSCs) have become a vital tool for modeling genetic diseases *in vitro* and have demonstrated rising importance in other applications as well. To be used for this purpose, they are usually patient-specific and differentiated into other cell types. They are then analyzed to determine the impact of the disease on development. Though they are typically used for diseases affecting other bodily systems such as in the cardiovascular and neurological fields, they have been shown to possess high potential for the musculoskeletal field. This area of study has typically been modeled using animal experiments or imaging techniques, and it is difficult to accurately model *in vitro*. Unfortunately, this leads to the neglect of musculoskeletal research involving techniques variant from the norm, more specifically the cellular mechanisms underlying disease development in the skeleton. Therefore, this study addressed the gap left by previous models through the development of an *in vitro* model of JOCD that focuses on the cellular impact the disease has on developing cartilage and bone.

# CHAPTER 2

# METHODS AND MATERIALS

## Cell Differentiation

Fibroblasts were extracted and expanded from the skin biopsies of 2 healthy and 3 JOCD pediatric subjects; viral reprogramming and cloning of these cells generated 3 iPSC control lines and 9 iPSC disease lines. The disease lines were reprogrammed at the University of Washington in St. Louis, and the control lines were gifted from the Regenerative Medicine Institute at the National University of Ireland Galway. Two additional control iPSC lines were purchased from ATCC to increase the number of control lines to 5. Characterization of iPSCs using IHC, teratoma formation, PCR, and embryonic body formation confirmed the pluripotency of the lines. Subsequent differentiation into mesenchymal differentiation (iMSCs) using induction media was confirmed using FACs analysis, PCR, and multipotency tests.

Using a 3-D pellet culture system, the iMSCs were divided into three groups and underwent an induction process during which they were fed media that contained specific growth factors to promote specific differentiation over several weeks of incubation. The pellets were cultured in round-bottom, 96-well plates, and the 200 μL of media per well was changed every other day, with the exception of weekends. They were fed media based on the schedule outlined in Figure 1. The first group was the control group, and it was fed basic MSC maintenance media throughout the culture period. The second group was fed media that promoted differentiation into cartilage (chondrogenesis). The third group was fed the same chondrogenic media for the first 21 days, but after this point, the media was switched to media that stimulated bone differentiation (osteogenesis).

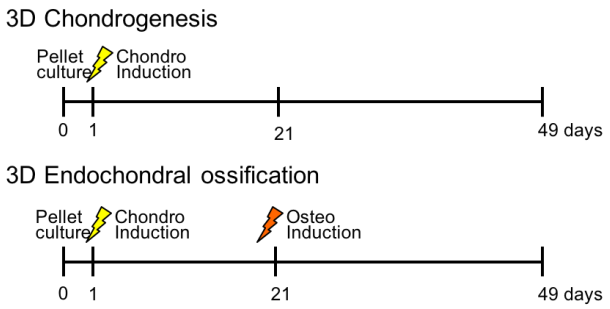


Figure 1: Schedule of the 3-D differentiation process. Chondro induction refers to feeding the organoids with chondrogenic media. Osteo induction refers to feeding the organoids with osteogenic media.

**Quantitative Analysis**

Pellets from each group were washed in DPBS and either snap frozen at -80 degrees Celsius until digestion or immediately digested at day 0, 7, 14, 21, 28, 35, and 49 to quantify differentiation and disease progression. In order to measure chondrogenic differentiation, a Glycosaminoglycan (GAG) Assay was conducted to quantify the sulfated proteoglycans and glycosaminoglycan content, an important component of cartilage extracellular matrix. The pellets were snap frozen until use, digested in a papain solution overnight, and homogenized before carrying out experiments. The results of the assay were then normalized with the DNA content found using a DNA Picogreen Assay with the same samples. To measure the osteogenic differentiation, the pellets were digested in acetic acid solution overnight, homogenized, and used for a Calcium Assay to analyze calcium deposition. These results were normalized with the DNA content from a DNA Picogreen Assay with samples harvested and digested in papain solution.

**Histological Analysis**

Several pellets from each group were collected at the time points mentioned previously and fixed in 10% Formalin solution overnight for qualitative analysis. Once fixed, they were embedded in 5% agarose, dehydrated in 70% ethanol, and submitted for paraffin processing. They were then embedded in paraffin, sectioned using a microtome, and stained to assess cell distribution and morphology. Hematoxylin-eosin (H&E) staining was used to stain the cytoplasm and DNA within the cell nuclei. Toluidine blue staining dyed the cartilage and nuclei to verify the results of the GAG Assays; Alcian blue was used to visualize the cartilage formation of the cells. Immunofluorescent staining was used to visualize the distribution of extracellular matrix (ECM) proteins such as collagen type 1 (Col1), collagen type 2 (Col2), and aggrecan.

# CHAPTER 3

# RESULTS

## Chondrogenic Differentiation

The GAG content of the chondrogenic organoids normalized by the DNA content is shown in Figure 2. There was no significant difference in GAG production between the control and JOCD pellets. This was found for both the day 21 time point and the day 49 time point. The data was analyzed using a two-way ANOVA with a one-way ANOVA post hoc for parametric data and a Kruskal-Wallis test for non-parametric data. Figure 3 shows the distribution of GAGs, appearing purple, within the pellets following staining with Toluidine Blue. As seen in Figure 4, the JOCD organoids appear to develop a shell of Col1 that is not present to the same degree in the normal organoid. The Aggrecan appears to be more concentrated in the center of the JOCD pellet compared to the control.

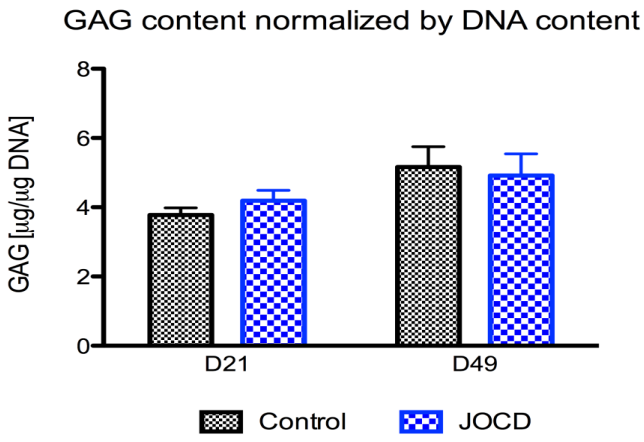


Figure 2: Level of GAG production measured as ug of GAG per ug of DNA. The results were shown at day 21 (D21) and day 49 (D49).

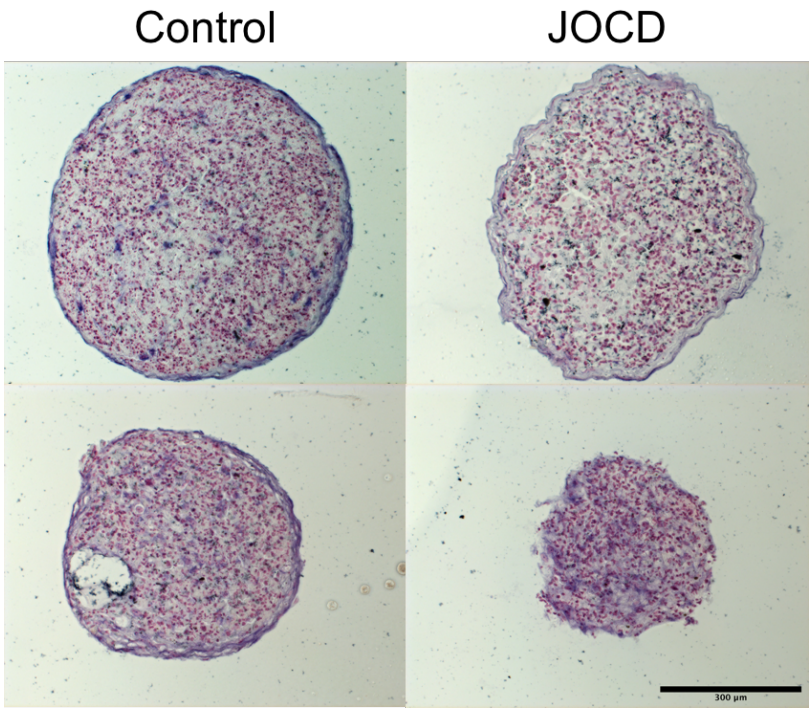


Figure 3: Toluidine Blue staining for GAG distribution in control and JOCD chondrogenic organoids. GAG is stained purple and cell nuclei appear dark pink. The images were taken at 20x magnification. The scale bar refers to 300um.

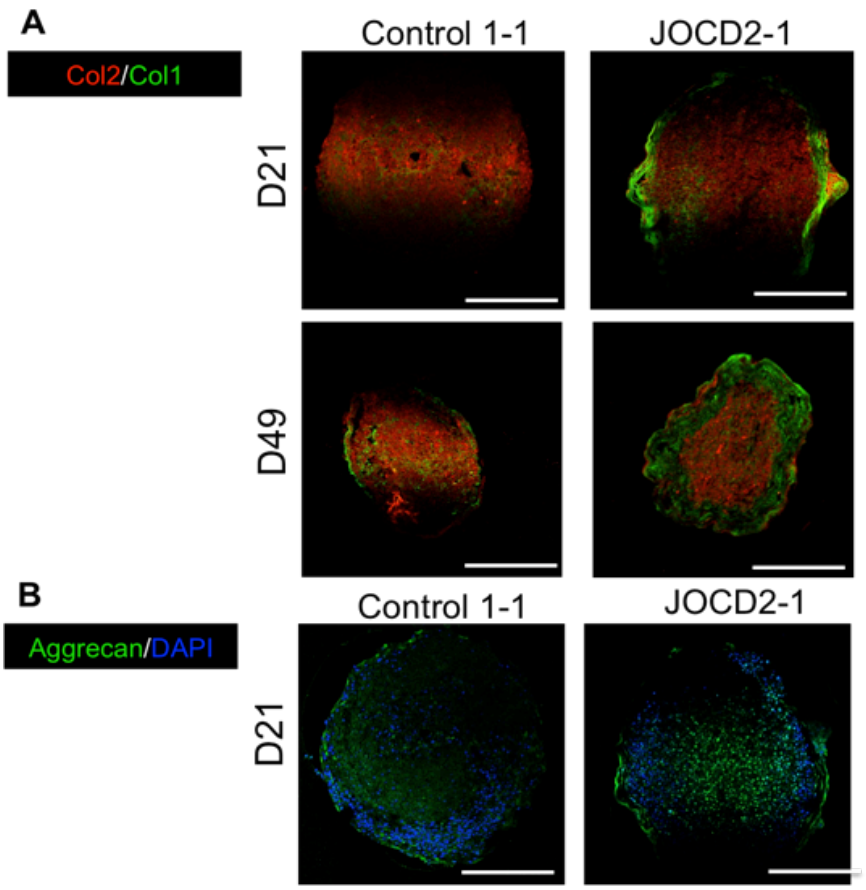


Figure 4: Immunostaining for ECM protein distribution in control and JOCD chondrogenic organoids. (A) Distribution of Col1 and Col2 in the organoids at day 21 and day 49. (B) Distribution of Aggrecan and cell nuclei (stained with DAPI) at day 21. The scale bars refer to 500um.

**Osteogenic Differentiation**

The calcium deposition of the osteogenic organoids normalized by the DNA content is shown in Figure 5. The statistics were run similarly to the chondrogenic organoids mentioned above, and significance was found at $p<0.001$. There was a significant difference between the day 28, day 35, and day 49 time points for both the JOCD and control groups. However, there was no significant difference between the JOCD and control groups at any of the time points.

The immunostaining in Figure 6 shows that the osteogenic JOCD organoids do not seem to develop the same Col1 shell that the normal organoids develop at day 49.

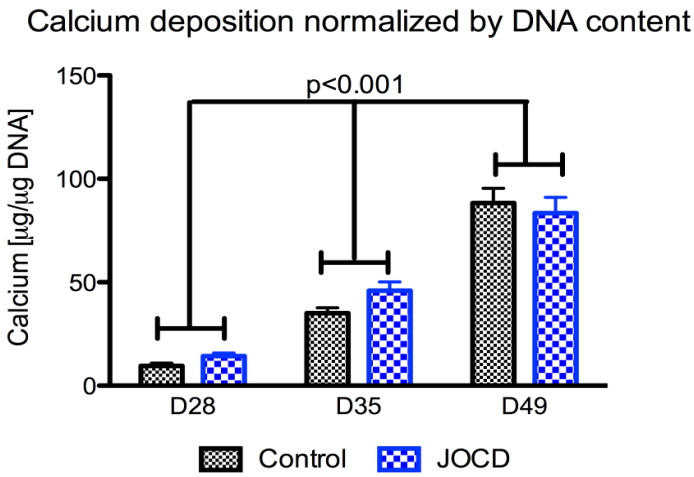


Figure 5: Level of calcium deposition measured as ug calcium per ug of DNA. Significance between time points was found as $p<0.001$.

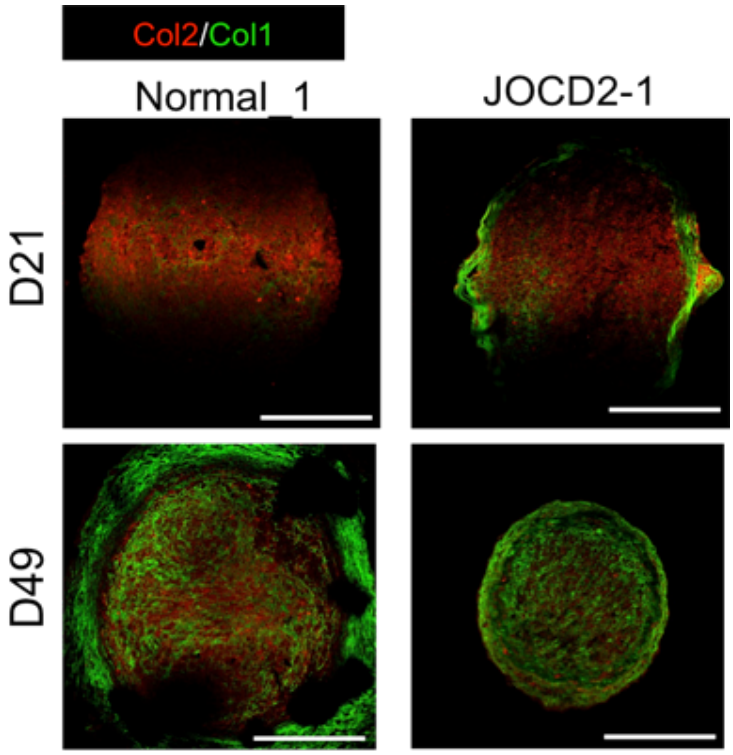


Figure 6: Distribution of Col1 and Col2 in control and JOCD osteogenic organoids at day 21 (D21) and day 49 (D49). The scale bars refer to 500um.

# CHAPTER 4

# DISCUSSION AND FUTURE WORK

The result of the GAG assay suggests that there is no statistical difference in the production and deposition rates of the extracellular matrix protein between groups (Figure 2). This could be due to the inconsistent GAG distribution relative to cell content between diseased pellets, as shown in Figure 3. The control pellets appear to demonstrate a consistent distribution of GAG relative to the number of cell nuclei. In contrast, one of the JOCD pellets appears to possess a level of GAG relative to the number of nuclei that is less than the control, while the other pellet seems to possess a much greater ratio than the control (Figure 3). This variation between diseased cell lines could have led to the inconclusive results of the GAG assay, but more research is needed to support this hypothesis. The immunostaining demonstrates an obvious difference in the distribution of Col1 and Col2 throughout the organoids, as the diseased state of JOCD seems to cause collagen type one to form a thick shell around the pellet (Figure 4). This could be due to the inability of the diseased cartilage to regulate their extracellular matrix deposition throughout the organoid.

The Calcium assay also suggests that there is no significant difference in the production and deposition rates of calcium between groups (Figure 5). There is a significant difference between time points, but this is an expected characteristic that simply helps to confirm the osteogenic differentiation of the organoids and gives no insight into the diseased state. However, similarly to the chondrogenic analysis, the staining demonstrated an obvious difference in the deposition distribution of extracellular matrix proteins (Figure 6).

Future work could focus on quantifying the gene expression of ECM proteins and deposited substances, such as GAG and calcium. Quantifying the gene expression of Col1, Col2,

and Aggrecan in both the osteogenic and chondrogenic groups could also prove useful. This could demonstrate whether the production of these proteins is dysregulated as well. The histological staining of the calcium distribution throughout the osteogenic organoids would prove useful in further understanding the disease pathogenesis as well. Additionally, the analysis or imaging of the endoplasmic reticulum could show whether JOCD had a similar mechanism of protein dysregulation through an engorged endoplasmic reticulum.

# CHAPTER 5

# CONCLUSION

These iPSC-based *in vitro* models of JOCD were able to recapitulate many aspects of the disease that allowed us to gain more understanding of the cellular pathogenesis. The quantitative analysis of several extracellular matrix deposits simply showed that the disease organoids were differentiating similarly to to the control, yet the qualitative analysis demonstrated the differences in the distribution as a result of the disease. This suggests that contrary to FOCD and previous understandings of the OCD family, the pathogenesis of JOCD may lie more in the distribution patterns of the extracellular matrix rather than the production rates. This novel understanding of the mechanisms by which the defective bone and cartilage develop would not have been possible without the use of these *in vitro* models, and they will further serve as a potential test bed to develop new treatments and progress deeper understanding of the OCD disease family.

# REFERENCES

Bruns, J., Werner, M., & Habermann, C. (2017). Osteochondritis Dissecans: Etiology, pathology, and imaging with a special focus on the knee joint. *Cartilage*, 1-17. doi:10.1177/1947603517715736

Edmonds, E. W., & Polousky, J. (2013). A review of knowledge in Osteochondritis Dissecans: 123 years of minimal evolution from Konig to the ROCK study group. *Clinical Orthopaedics and Related Research, 471*(4), 1118-1126. doi:10.1007/s11999-012-2290-y

Garvican, E. R., Vaughan-Thomas, A., Redmond, C., & Clegg, P. D. (2008). Chondrocytes harvested from osteochondritis dissecans cartilage are able to undergo limited in vitro chondrogenesis despite having perturbations of cell phenotype in vivo. *Journal of Orthopaedic Research, 26*(8), 1133-1140. doi:10.1002/jor.20602

Miyanishi, K., Trindane, M. C. D., Linsey, D. P., Beaupre, G. S., Carter, D. R., Goodman, S. B., . . . Smith, R. L. (2006). Dose- and time-dependent effects of cyclic hydrostatic pressure on transforming growth factor-beta3-induced chondrogenesis by adult human mesenchymal stem cells in vitro. *Tissue Engineering, 12*.

Park, I. H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., . . . Daley, G. Q. (2008). Disease-specific induced pluripotent stem (iPS) cells. *Cell, 134*(5), 877-886. doi:10.1016/j.cell.2008.07.041

Pfeifer, C. G., Kinsella, S. D., Milby, A. H., Fisher, M. B., Belkin, N. S., Mauck, R. L., & Carey, J. L. (2015). Development of a large animal model of Osteochondritis Dissecans of the knee: A pilot study. *The Orthopaedic Journal of Sports Medicine, 3*(2). doi:10.1177/2325967115570019

Saha, K., & Jaenisch, R. (2009). Technical challenges in using human induced pluripotent stem

cells to model disease. *Cell Stem Cell, 5*(6), 584-595. doi:10.1016/j.stem.2009.11.009

Salazar-Noratto, G. E., Barry, F. P., & Guldberg, R. E. (2016). Application of biomaterials to in vitro pluripotent stem cell disease modeling of the skeletal system. *Journal of Materials Chemistry B, 4*, 3482-3489. doi:10.1039/c5tb02645h

Wu, S. M., & Hochedlinger, K. (2011). Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nature Cell Biology, 13*(5), 497-505. doi:10.1038/ncb0511-497

Xu, M., Stattin, E. L., Shaw, G., Heinegard, D., Sullivan, G., Wilmut, I., . . . Barry, F. (2016). Chondrocytes derived from mesenchymal stromal cells and induced pluripotent cells of patients with Familial Osteochondritis Dissecans exhibit an endoplasmic reticulum stress response and defective matrix assembly. *Stem Cells Translational Medicine, 5*(9), 1171-1181. doi:10.5966/sctm.2015-0384